

Altered antigenicity of 'a' determinant variants of hepatitis B virus

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The 'a' determinant of hepatitis B virus (HBV) surface antigen (HBsAg) is the most important target for diagnosis and immunoprophylaxis. Several HBV variants with point mutations within the 'a' determinant have been identified among fully vaccinated children in Taiwan. We investigated the effect of each of these mutations on the antigenic nature of the S protein by cloning and expression of the mutant S antigens in *Pichia pastoris*. Four variants, Ser-126, His-129, Arg-129 and Arg-145, all exhibited various degrees of altered binding of HBsAg to several monoclonal antibodies. Arg-145,

a well-characterized immune escape mutant, and Arg-129 had the lowest binding capacities to all monoclonal antibodies as compared with other variants. Similar to Arg-145, the Arg-129 variant could be isolated from both vaccinated children and unvaccinated adults, thus representing a naturally occurring mutant with an altered 'a' determinant. Whether these 'a' determinant variants with altered antigenicity might gradually become major circulating strains, as a consequence of the immune pressure against the wild-type HBV created by vaccination, remains to be monitored.

Introduction

Hepatitis B viruses (HBV) share a common immunodominant and immunoprotective determinant on their surface antigen, amino acids (aa) 124 to 147 on the small S protein, which is termed the 'a' determinant. This 24 amino acid 'a' determinant contains five cysteine residues which putatively form cysteine-disulfide bonds to yield two cyclized antigenic loops (Brown *et al.*, 1984; Guerrero *et al.*, 1988; Howard *et al.*, 1988). *In vitro* studies have shown that antigenicity is lost when the cysteine residue at aa position 124, 137, 139, 147 or 149 is substituted with serine, suggesting that these amino acids are critical for expression of the full antigenicity, whereas the same mutation of the cysteine residue at position 138 has little or no effect upon antigenicity (Antoni *et al.*, 1994; Ashton-Rickardt & Murray, 1989; Bruce & Murray, 1995; Mangold & Streeck, 1993). Therefore, it is believed that neutralizing antibodies induced by the HBV vaccine are largely targeted towards conformational epitopes (Wands *et al.*, 1984).

An arginine-145 (Arg-145) variant, which contains arginine, rather than glycine, at aa position 145 of the HBV S antigen, has been found in individuals who have received HBV

vaccine and/or been treated with immunoglobulin against HBV (Carman *et al.*, 1990; Fujii *et al.*, 1992; Harrison *et al.*, 1991; Hino *et al.*, 1995; Ho *et al.*, 1995; Moriyama *et al.*, 1991; Okamoto *et al.*, 1992; Oon *et al.*, 1995), in liver transplant patients receiving monoclonal antibody against HBV (McMahon *et al.*, 1992), and in both unvaccinated and untreated adults (Chirara & Chetsanga, 1994; Kohno *et al.*, 1996; Yamamoto *et al.*, 1994). This Arg-145 variant has a much lower binding capacity to the antibodies (anti-HBs) raised against wild-type HBV (glycine-145) (Waters *et al.*, 1992) and, therefore, is an immune escape mutant. Other mutations within the 'a' determinant have also been described, e.g. mutations at aa 126 (Okamoto *et al.*, 1992; Yamamoto *et al.*, 1994), aa 129 (Oon *et al.*, 1995; Zhang *et al.*, 1996), aa 130 (Kohno *et al.*, 1996; Zhang *et al.*, 1996), aa 141 (Fortuin *et al.*, 1994; Howard *et al.*, 1994; Karthigesu *et al.*, 1994) and aa 144 (Harrison *et al.*, 1994; Ni *et al.*, 1995; Oon *et al.*, 1995; Wallace *et al.*, 1994). However, the immunological, clinical and epidemiological significance of most of these HBV mutants have not been well characterized.

In this study, we comparatively evaluated the impact of amino acid substitutions within the 'a' determinant on the antigenicity of the S protein *in vitro*. Each of the four selected HBV isolates represented an altered physical or chemical property of one amino acid substitution within the 'a'

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determinant. Amino acid substitution at aa 126 has been thought to have little impact on antigenicity (Kohno *et al.*, 1996). The Arg-145 variant represents a well-characterized immune escape mutant (Waters *et al.*, 1992) and, therefore, is included for comparative purposes. The other two variants both have a point mutation at aa position 129 (Arg-129 and His-129). Arg-129 represents a drastic change in molecular mass from 146 (glycine) to 174 and a switch from a neutral to a positive charge, whereas His-129 represents a lesser change in molecular mass, from 146 (glycine) to 155, and a switch from a neutral charge to a polar state.

Methods

■ **Selection of the HBV variants.** HBV carrier children were identified through a sampling survey among children who had been fully vaccinated under a national children's mass immunization programme (Hsu *et al.*, 1996). DNA sequence analysis of the gene encoding HBV surface antigen was carried out for the total 64 HBV isolates harboured among the HBsAg seropositive children. The five HBV isolates were selected because they represent a spectrum of the switch in the properties of amino acid residues within the 'a' determinant.

■ **Amplification and sequence analysis of S genes.** DNA was extracted from human serum by proteinase K treatment and the phenol-chloroform method (Sambrook *et al.*, 1989). The S gene of HBV, flanking a 681 bp fragment, was amplified from these DNA samples with primers BV52 (5' CGAATATGGAGAACATCACA) and BV32 (5' G-TTAAATGTATACCCAGAGAC). The amplicon was then cloned into a pCR II vector (Invitrogen).

■ **Preparation of recombinant HBsAg.** After DNA sequence verification by the fluorescence-based Sanger dideoxy sequencing method (DyeDeoxy Terminal Taq Sequencing Kit; Applied Biosystems), the full-length S genes of the selected variants were subcloned into an expression vector, pHIL-D2 (Invitrogen), which used the AOH1 promoter. The S gene-pHIL-D2 construct was integrated into the genome of *Pichia pastoris* by a one-step gene replacement method (Rothstein, 1983) to yield S antigen-expressing strains. To induce the S antigen (termed rHBsAg) expression, 1% methanol was used as the sole carbon and energy source in shake-flask cultures of the recombinant strain of *Pichia pastoris*. After 4 days of methanol induction, the yeast cells were pelleted and disrupted with microfluidizer (model 110S; Microfluidics Corp.), and the cellular debris was eliminated by centrifugation. The rHBsAg was purified from cell lysate by serial ultracentrifugation in caesium chloride and sucrose gradients (Cregg *et al.*, 1987). The gradient fractions containing HBsAg were identified by a sandwich ELISA assay (Surase B-96 EIA, General Biologicals Corp.) which utilized a monoclonal anti-HBs antibody of the IgM type to capture rHBsAg and a conjugated polyclonal anti-HBs from guinea-pig to detect HBsAg. All positive fractions were pooled and dialysed against PBS overnight. The total protein content was measured using a bicinchoninic acid (BCA) protein assay (Smith *et al.*, 1985). For comparative purposes, another recombinant HBsAg which had been expressed in *Saccharomyces cerevisiae* (termed S-SC hereafter; gift from Developing Center of Biotechnology, Taipei, Taiwan) was tested along with our 'wild-type' rHBsAg from *Pichia pastoris* (termed S-PP hereafter).

■ **Binding of variant antigens to a guinea-pig-derived polyclonal antibody.** ELISA plate wells were coated with a serial dilution of rHBsAg and blocked with 8% non-fat milk. An excess amount of guinea-

pig-derived polyclonal anti-HBs conjugated with horseradish peroxidase (HRPO) was allowed to bind with the rHBsAg coated on the plate. Extensive washing with PBS was carried out between each step and before OPD was added as substrate. The A_{490} values, which reflected the amount of rHBsAg on the plate, were read. To standardize the amount of the variant rHBsAg, a concentration of each sample resulting in an A_{490} value of 1 (indicated by the arrow in Fig. 3a) by the above polyclonal binding assay was used in all subsequent monoclonal antibody binding assays. The concentration of the rHBsAg was also determined by an ELISA along with a standard curve made up with a known HBsAg concentration as provided in a commercial ELISA kit (Surase B-96 EIA, General Biologicals Corp.). The antigenic equivalence of HBsAg contained in rHBsAg was subsequently adjusted to the same level.

■ **Binding of the variant antigens to a panel of anti-HBs monoclonal antibodies.** The binding kinetics between each rHBsAg and a panel of the 'a' determinant-specific monoclonal antibodies which included 1B5 and 1B6 (Bioscience Inc.), 2A7 (Abbott), 3E7 (Dako) and 4H5 (Fitzgerald Inc.), were tested in the liquid phase. Each rHBsAg (containing an equivalent ng quantity of HBsAg) was incubated with a serial dilution of monoclonal anti-HBs overnight at room temperature. The unbound monoclonal anti-HBs was quantified by an ELISA assay (Antisurase B-96 EIA, General Biologicals Corp.) which used human plasma-derived HBsAg as an antigen to capture the antibody. Plasma-derived HBsAg-HRPO conjugate was used to quantify the captured monoclonal anti-HBs. The ability of each rHBsAg to bind with each monoclonal antibody was inversely correlated with the absorbance readings. The resultant reading was compared with that of the identical dilution of monoclonal antibody without binding to the rHBsAg so that the percentage blocking may be calculated.

Results

Based on the amino acid substitution occurring within the 'a' determinant, four HBV variants of the adw2 subtype were selected: threonine to serine at aa 126 (Ser-126), glutamine to histidine (His-129) or to arginine (Arg-129) at aa 129, and glycine to arginine at aa 145 (Arg-145). In addition, one adw2 wild-type without mutation within the 'a' determinant (S-PP) was also included in this study for comparison. Multiple clones of the entire S gene of each variant were selected for sequence analysis, and the clone representing the consensus sequence of the S gene variant was selected to be subcloned into the expression vector. The amino acid sequences of the entire S antigen as deduced from DNA sequence analysis of these variants indicated that a number of amino acid substitutions also occur outside of the 'a' determinant: five for Arg-129, four for Ser-126, two for Arg-145 and none for His-129 (Fig. 1).

The recombinant HBsAg (termed rHBsAg hereafter) was prepared and partially purified for further characterization by monoclonal antibody binding assays. To compare the *Pichia pastoris*-expressed rHBsAg with the HBsAg derived from plasma of HBV-infected human subjects and with the rHBsAg expressed in *Saccharomyces cerevisiae* (S-SC), we studied the binding kinetics of these proteins with polyclonal antisera (derived from guinea-pig) following the capture of these proteins by a monoclonal antibody of the IgM type. We noted that the S-PP and S-SC rHBsAg had an 'a' determinant

	1	50	51	100
S-PP	g
Ser-126	e.g
His-129
Arg-129	f.....	s.....
Arg-145
Consensus	MEKITSGLLG	PLVLQAGFP	LLTEILTIPQ	SLDSWTSLN
			PLGCTPVCLA	QNSQSQISSH
			SPTCCPPICP	GYRWMLRRF
			HFICILLLC	LIFLLVLDY
	101	150	151	200
S-PP
Ser-126	v.....
His-129
Arg-129
Arg-145
Consensus	QGMLPVCPLI	PGSSTTSTGP	CKTCTPAQG	TSKPPSOCT
			KPTDGNCTCI	PIPSSWAPAK
			YLWEVASVRF	SWLSLVVVF
			QTFVGLSPTV	WLSVIVWVY
	201	227		
S-PP		
Ser-126		
His-129		
Arg-129		
Arg-145		
Consensus	WGPSLYNLS	PPIPLPIFP	CLWVYI-	

Fig. 1. Amino acid sequences of five HBsAg deduced from nucleotide sequences of HBV variants derived from vaccinated children and unvaccinated adults. The single-letter code for amino acid is used in the consensus sequence; homologous amino acids are indicated with a dot, and heterologous amino acids are specified. The numbers on the top indicate the amino acid position in the major S antigen. The underlined sequences are the 'a' determinant of the S antigen.

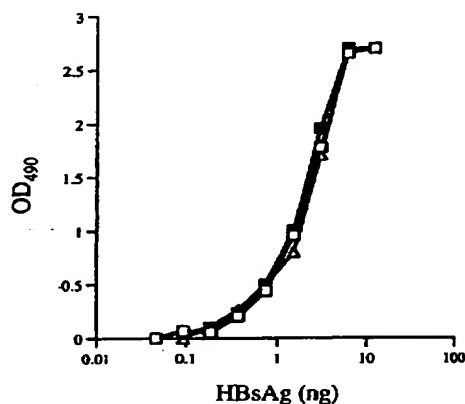


Fig. 2. Binding kinetics between a guinea-pig-derived polyclonal antibody and three HBsAg preparations, plasma-derived HBsAg, recombinant HBsAg derived from *Pichia pastoris* (termed S-PP) and recombinant HBsAg derived from *Saccharomyces cerevisiae* (termed S-SC). The amount of HBsAg indicated on the horizontal axis was measured against a standard amount of HBsAg as provided in a commercial kit (Surase B-96 EIA, General Biologicals Corp.). □, plasma-derived native HBsAg; ■, rHBsAg (S-SC); △, rHBsAg (S-PP).

consensus sequence identical to that of the wild-type, and shared identical binding kinetics to the polyclonal antisera as plasma-derived HBsAg (Fig. 2). Furthermore, these three HBsAg preparations also had similar binding kinetics to one monoclonal antibody (2A7) which had been conjugated with HRPO so that performance of a similar binding assay was

possible. These data provided a basis for valid comparison in subsequent assays.

The antigenic equivalence of HBsAg contained in each preparation of the rHBsAg was determined by immunoassay using a guinea-pig-derived polyclonal antibody. The concentration of each rHBsAg preparation was adjusted to the same antigenic equivalence of HBsAg so that the binding kinetics of these variant rHBsAg to the guinea-pig-derived polyclonal antibody could be compared among one another (Fig. 3a). While the Ser-126 and His-129 variants had binding kinetics similar to that of S-PP of the wild-type, the Arg-129 and Arg-145 variants were remarkable for the peak absorbance reading being limited to under 2, suggesting a saturated binding capacity of Arg-129 or Arg-145 variants to the polyclonal antisera. Furthermore, the total protein concentration in the Arg-129 or Arg-145 preparations (as measured by a BCA method) with the same antigenic equivalence of HBsAg was many fold higher than in preparations of other variants and the wild-type (Fig. 3b).

Using a competitive assay, we compared the ability of these rHBsAg variants to displace the plasma-derived wild-type HBsAg for binding to a panel of five monoclonal antibodies raised against the 'a' determinant of wild-type HBsAg (Fig. 4a-e). The Arg-129 and Arg-145 variants showed almost no binding to three of the five monoclonal antibodies tested, as indicated by nearly identical binding kinetics of these monoclonal antibodies to the plasma-derived HBsAg with or without blocking by the Arg-129 and Arg-145 variants (Fig.

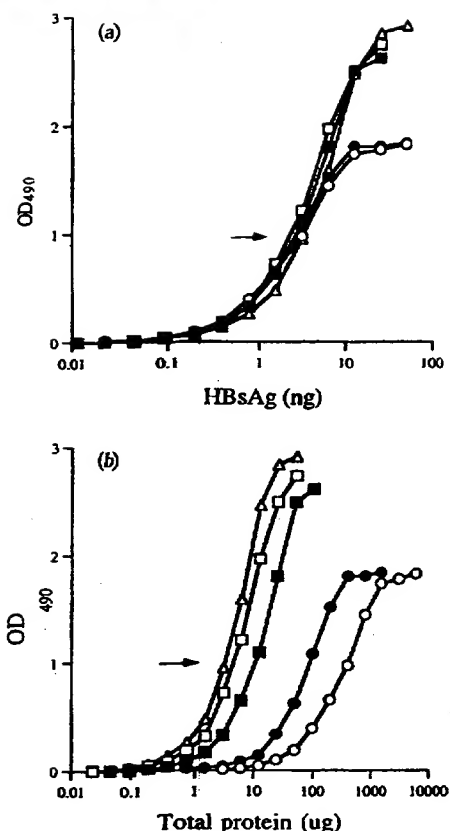


Fig. 3. (a) Determination of antigenic equivalence of HBsAg using a guinea-pig-derived polyclonal antibody. The arrow indicates a concentration of each sample resulting in an absorbance value around 1 which was chosen to calculate the antigenic equivalence of HBsAg of these variant rHBsAg preparations by comparison with that of S-PP. (b) Binding of a guinea-pig-derived polyclonal antibody by five *Pichia pastoris*-derived variant rHBsAg on the solid phase. ○, Arg-129; ●, Arg-145; □, His-129; ■, Ser-126; △, S-PP.

4c-e). Furthermore, the Arg-129 and Arg-145 variants showed lower binding to all five monoclonal antibodies compared with all other rHBsAg variants (Fig. 4a-e). Conversely, the binding kinetics of the His-129 rHBsAg variant were nearly identical to those of the wild-type rHBsAg for four of the five monoclonal antibodies (Fig. 4a, c, d, e), and the binding ability of the Ser-126 variant to all five monoclonal antibodies fell in between the above two extremes.

For quantitative comparison, the ability of each variant rHBsAg to block the binding of plasma-derived HBsAg to each monoclonal antibody was compared with the extent of blocking achieved by S-PP of the wild-type rHBsAg to a fixed dilution of each monoclonal antibody (as indicated by the arrows in Fig. 4a-e), which was taken to be 100% blockage. The derived ratio of the blocking percentage for variant HBsAg to that of S-PP is termed the 'similarity index'. Based on the average of two experiments, within the linear range of the binding kinetics where dilution of the monoclonal antibody

yielded an absorbance value around 1 (ranging from 0.7 to 1.1) without blocking, the similarity indices can be precisely calculated (Table 1). A similarity index of 1 signifies identity of the variant HBsAg to the wild-type HBsAg at the epitope corresponding to the monoclonal antibody being tested. Since no crossover occurs within the linear range of the binding kinetics of all the rHBsAg to each monoclonal antibody, the similarity indices showed a consistent pattern in terms of the relative order of magnitude for each rHBsAg variant. The similarity index was the lowest for Arg-129 and Arg-145 variants for binding to almost all the monoclonal antibodies, and was near 1 for the His-129 variant for binding to four of the five monoclonal antibodies. Ser-126 fell somewhere in between.

Discussion

Vaccine failure may be due to multiple factors. Apart from deficient host immunological status, neonatal tolerance and other elements related to the method of administration and the quality of vaccines, the altered antigenicity of an HBV variant may arise from one of the many contributing causes. While most of the 'a' determinant variants probably existed before the vaccination era (Carman *et al.*, 1990; Chirara & Chetsanga, 1994; Fujii *et al.*, 1992; Ho *et al.*, 1995; Kohno *et al.*, 1996; Okamoto *et al.*, 1992; Yamamoto *et al.*, 1994; Zhang *et al.*, 1996), widespread use of HBV vaccine has highlighted the importance of these variants because of the theoretical consideration that antibodies directed against the HBV wild prototype might potentially pose a selective advantage in favour of these variants.

From a systematic epidemiological study, several HBV variants were identified among the vaccinees in whom the HBV vaccine had failed to provide protection. Comparison between HBV viruses from vaccinated children and unvaccinated adults showed that 20% of all HBV isolates among the children and 28% of HBV isolates from the adults carried at least one point mutation within the 'a' determinant (M.-S. Ho, unpublished data). We found no evidence that the absolute number of 'a' determinant variants had increased during the first 7 years since the inception of the HBV vaccination programmes. Although multiple 'a' determinant variants have been reported (Kohno *et al.*, 1996; Oon *et al.*, 1995; Yamamoto *et al.*, 1994; Zhang *et al.*, 1996), the antigenic impact of these mutations has not been systematically studied.

In this study, the selection of HBV isolates was intended to cover a range of antigenic variation based on theoretical considerations concerning the nature of the amino acid substitution. The Arg-129 variant, which represents an altered hydrophobicity profile, would be expected to affect the secondary and tertiary structures of the antigen much more than in the His-129 variant, and our data supported such a hypothesis. The Ser-126 variant, initially intended to represent a minimal change in antigenicity (Kohno *et al.*, 1996), had a

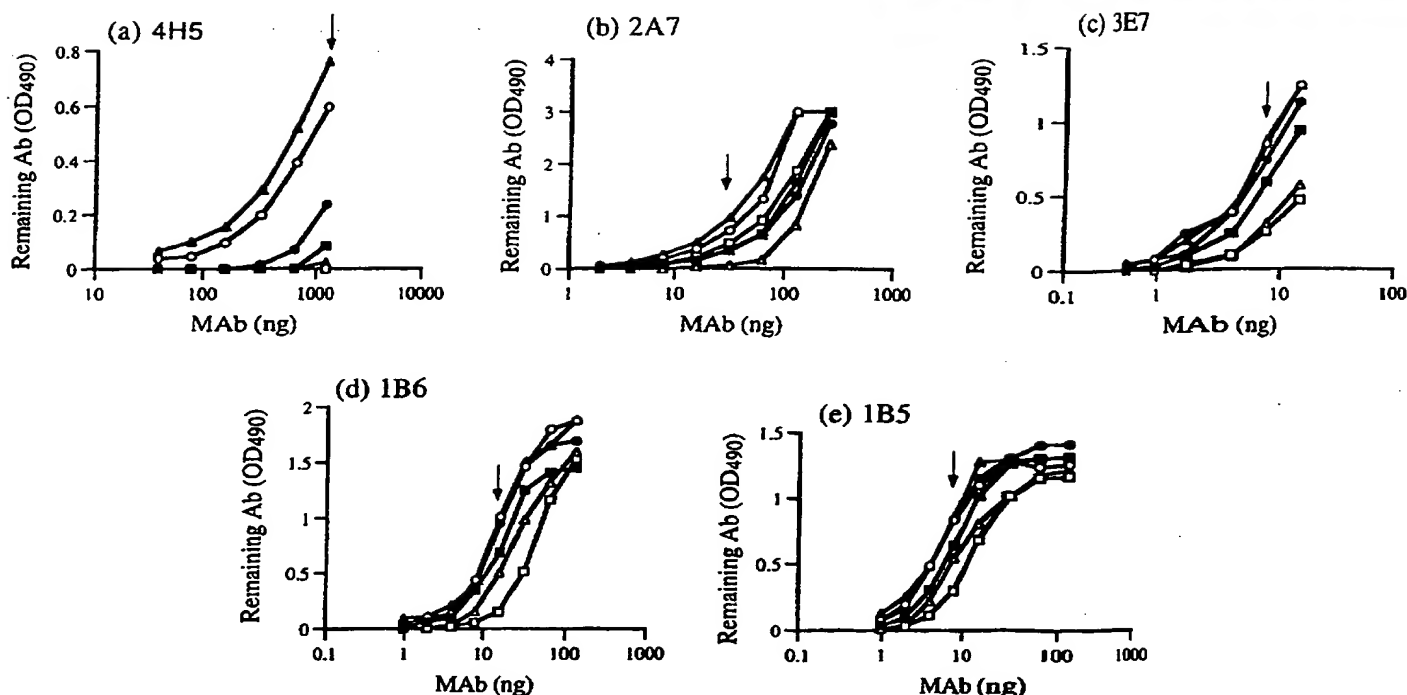


Fig. 4. Binding kinetics of plasma-derived HBsAg on the solid phase to five rHBsAg-preblocked monoclonal antibodies; the monoclonal antibodies are 4H5, 2A7, 3E7, 1B6 and 1B5. The arrows indicate the constant dilution of each monoclonal antibody which was selected for calculation of the index in Table 1. ○, Arg-129; ●, Arg-145; □, His-129; ■, Ser-126; △, S-PP; ▲, no blocking.

Table 1. Similarity index of the variant HBsAg versus the wild-type HBsAg at the epitope corresponding to the monoclonal antibody

The index was calculated by dividing the blocking percentage for variant HBsAg by that of S-PP rHBsAg (average of two experiments).

Monoclonal antibody	Similarity index			
	Ser-126	His-129	Arg-145	Arg-129
4H5	0.92	1.00	0.71	0.23
2A7	0.70	0.55	0.68	0.28
3E7	0.51	1.00	0.25	0.06
1B6	0.64	1.30	0.12	0.00
1B5	0.68	1.30	0.05	0.08

reduced binding capacity to several monoclonal antibodies. Residue 126, like residue 145, is located one amino acid away from a cysteine residue which is the putative site of disulfide bond formation, and the importance of residue 145 is well established (Waters *et al.*, 1992). It is plausible that residue 126 may similarly contribute to the stable establishment of disulfide bonds, which are crucial to the formation of a double-loop

structure of the 'a' determinant (Okamoto *et al.*, 1992). However, the caveat about this particular Ser-126 variant is that it also contains five other amino acid substitutions outside of the 'a' determinant, including valine for alanine at position 159. Amino acid 159 is important in the q — phenotype and plays a role in the expression of w1 reactivity (Norder *et al.*, 1992). How much of the altered antigenicity is contributed by Val-159 rather than Ser-126 is unknown for the time being since we have no suitable variants available for such comparison, but this warrants further investigation.

The identity of the binding kinetics to the polyclonal and monoclonal antibodies of the two yeast-derived rHBsAg and the plasma-derived native HBsAg provides a valid basis for the comparative analyses. Since the rHBsAg used in all assays was only partially purified, the higher concentration of total protein required to yield the same antigenic equivalence of HBsAg by the polyclonal binding assay could reflect differences in the degree of purity of each rHBsAg preparation. However, in all competitive assays, the rHBsAg concentration was adjusted to the same antigenic equivalence of HBsAg determined by the polyclonal binding; thus the problem of impurity is believed to be minimized. Alternatively, a lower avidity to the polyclonal antibody, or a lower affinity to each monoclonal antibody, might also be the contributing factor. Our data showing a total lack of or reduced binding ability of the Arg-129 and Arg-145

variants to all the monoclonal antibodies tested would indicate an altered affinity of these variants to these antibodies.

The lower maximal binding capacity of the rHBsAg Arg-129 and Arg-145 variants to the polyclonal antisera is most likely due to a loss of certain epitopes within the 'a' determinant. The inability of these two variant rHBsAg to bind with three of the five monoclonal antibodies corroborates the above hypothesis. The protection offered by immunity elicited by HBV vaccine, which is usually polyclonal in nature, may not be totally lost or severely affected *in vivo* by the substitution of only one amino acid. However, some individuals are known to have a more narrowly focused host immune response than others (Chisari & Ferrari, 1995; Missale *et al.*, 1993; Penna *et al.*, 1992). In these individuals, any loss of the limited responsive repertoire of the viral epitopes could accentuate the ability of the variant virus to evade the host immunity. The results of our study support the consideration that the 'a' determinant variants, which usually possess an altered antigenicity, could potentially evade immunity in individuals with a narrower scope of response to the current HBV vaccine. Although it has not been clearly demonstrated that these mutants have spread among vaccinated individuals so far (Whittle *et al.*, 1991), the potential for widespread use of HBV vaccine among a population to pose a selection pressure against the wild-type HBV and for the relative frequency of variants (Arg-145 currently being 1/64 and Arg-129 being 2/64), to increase with time should be monitored. Since HBV vaccine is the only effective means currently available to prevent non-parental transmission of HBV, a main contributor to maintenance of the hyperendemicity of HBV in many parts of the world, the long-term impact of these HBV variants should be actively monitored in order to ensure the continued success of HBV control through immunization.

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